Early life exposure to toxic environments: effects on lung and immune cell development in mice and men

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Chapter 3

Smoking during pregnancy inhibits ciliated cell differentiation and upregulates secretory cell-related genes in neonatal offspring

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Submmited

Abstract: Maternal smoking during pregnancy is an independent risk factor for children to develop asthma and respiratory infections. We have recently shown that maternal smoking during pregnancy increased house dust mite-induced goblet cell metaplasia in an experimental mouse model. To further study the effects of prenatal smoke exposure on airway epithelial cell development in neonatal offspring, female C57BL/6 mice were exposed to fresh air or cigarette smoke from 1 week prior to conception until birth. Offspring was all sacrificed 1 day after birth. In lung tissue, numbers of ciliated cells and secretory club cells were investigated by immunohistochemistry, as well as mRNA expression of transcription factors that regulate ciliated cell and secretory cell differentiation. We found that maternal smoking inhibited the number of ciliated cells, as well as expression of the major cilia-related transcription factor Forkhead box J1 (Foxj1) in offspring. In addition, increased expression of transcription factors involved in secretory cell differentiation, such as Forkhead box M1 (Foxm1) and Sam pointed domain-containing ETS transcription factor (Spdef) was found in offspring from the smoke-exposed mothers. This was accompanied by higher mRNA expression of Hairy/enhancer-of-split related with YRPW motif protein 1 (Hey1), a Notch target gene. Induced expression of Hey1 suggests that Notch signaling may be involved in the observed aberrant epithelial cell development after prenatal smoke exposure. The lower number of ciliated cells could affect mucociliary clearance and may explain the increased susceptibility of children from smoking parents to wheeze and to development of childhood respiratory infections.

Introduction

The trachea and main bronchi are lined by a pseudostratified epithelium that is composed of ciliated cells, club cells (formally known as Clara cells) and basal cells (Crystal, et al. 2008; Herriges and Morrisey. 2014) as major cell types. In humans, the basal cells underlie the ciliated and secretory (club and goblet) cells. A similar epithelial architecture is present in the mouse, although it is limited to

the trachea and the largest bronchi. Ciliated and club cell types act as the front-line defense in a coordinated manner to protect the lungs from inhaled pathogens and noxious agents. Thus club cells secrete a layer of mucus, trapping inhaled harmful particles and pathogens, whereas ciliated cells clear the latter from the airways by cilia beating, generating a one way wave-like movement across the epithelial surface (Knowles and Boucher. 2002). Lineage studies in mice and *in vitro* suggest that most lung epithelial cell lineages have (self-)renewal capacity and (re)generate secretory and ciliated epithelial cell phenotypes both during development and in response to injury (Park, et al. 2006; Sun, et al. 2013; Kotton and Morrisey. 2014). This process is controlled in part by Notch signaling, which promotes secretory cell fate and inhibits ciliary cell differentiation (Guseh, et al. 2009; Rock, et al. 2009; Rock, et al. 2011). In addition, goblet cell metaplasia is dictated by the transcription factors Spdef, Foxm1, Foxa2 and Nkx2.1 (Park, et al. 2007; Maeda, et al. 2011b; Ren, et al. 2013) whereas development of ciliated cells is driven by Foxj1 (Brekman, et al. 2014). Of note, murine goblet cells are derived from club cells and arise only after injury or in disease states (Pardo-Saganta, et al. 2013).

In an experimental mouse model for asthma, we have previously shown that smoking during pregnancy substantially increased goblet cell numbers in the airways of 10-week-old offspring after 5 weeks of house dust mite (HDM) exposure (Blacquiere, et al. 2009). This observation is of interest as it is in line with several birth-cohort studies in which maternal smoking during pregnancy was shown to be a risk factor for the development of transient early wheeze (Civelek, et al. 2011; Caudri, et al. 2013) as well as asthma, extending into adulthood (Civelek, et al. 2011; Caudri, et al. 2013; Grabenhenrich, et al. 2014). In addition, children born from mothers who smoked during pregnancy have a higher risk to develop respiratory infections (Broughton, et al. 2005).

In the current study, we therefore aimed to get more insight into epithelial cell differentiation and gene expression, directly after birth, in prenatally smoke-exposed mice, to explain enhanced goblet cell differentiation susceptibility later in life. This was addressed firstly by analyzing the number of ciliated cells and club cells in the conducting airways and secondly by analyzing

the expression of genes that dictate proper epithelial cell differentiation.

Material and Methods

Animals

Female and male C57BL/6 mice, aged 8-10 weeks, were obtained from Harlan (Horst, The Netherlands). Mice had access to standard food and water ad libitum. The animal study was approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 6589a) and was performed under strict governmental and international guidelines on animal experimentation.

Cigarette smoke exposure

Mainstream cigarette smoke was generated using a TE-10 smoke exposure system of Teague Enterprises Smoke Exposure System (Woodland, California, USA). Female mice were exposed to fresh air (n=3) or cigarette smoke (n=4) in two sessions of 50 minutes with a 3h interval between both exposures per day in which smoke of 10 cigarettes were generated per session. Mice were exposed from 7 days before mating until the day of sacrifice. The adaption protocol included exposure to 3 cigarettes per session the first day, 5 cigarettes the second day, 7 cigarettes the third day and 10 cigarettes the fourth day and thereafter. Smoking 10 cigarettes in one session generated total particulate matter counts of at least 200 mg/m^3 and a CO level of 250 PPM (max). Kentucky 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, Kentucky, USA) were used. For experimental purposes, female mice were treated with 1.25 IU pregnant mare's serum gonadotrophin and 1.25 IU human chorionic gonadotrophin to induce simultaneous cycling. To induce pregnancy, 1 female was housed with 1 male. Mating was confirmed by vaginal plug detection. Smoke exposure remained constant during the total pregnancy. Mothers and offspring were not exposed to cigarette smoke after offspring was born. Offspring (n=16 from non-smoking mothers, n=24 from smoking mothers) was sacrificed one day after birth. The left lung was used for qRT-PCR analyses. The right lung was fixed in 4%

paraformaldehyde and embedded in paraffin for immunohistochemical analysis.

qRT-PCR analysis in lung tissue

Total RNA was isolated from lung tissue using a RNA isolation trizol kit (Invitrogen, USA). cDNA was reverse transcribed using a Superscript-II Reverse Transcriptase kit (Invitrogen, USA). To measure the expression of Gapdh (Mm99999915_g1), Foxj1 (ciliated cell, assay ID: Mm01267279_m1), Scgb1a1 (Cc10, Club cell, Mm00442046_m1), Calca (neuroendocrine cell, Mm00801463_g1), Trp-63 (p16, basal cell, Mm00495793_m1), and Keratin 5 (krt5, basal cell, Mm01305291_g1), Foxm1 (Mm00514924_m1), Spdef (Mm00600221_m1) and Muc5ac (Mm01276718_m1), Foxa2 (Mm01976556_s1), Foxa3 (Mm00484714_m1), Nkx2.1 (Mm00447558_m1), Hoxa5 (Mm04213381_s1) and Hoxb5 (Mm00657672_m1), Notch1(Mm00435249_m1), Notch2 (Mm00803077_m1), Notch3 (Mm01345646_m1), Hey1 (Mm00468865_m1), Hey2 (Mm00469280_m1), Hes1 (Mm01342805_m1), on demand Gene Expression Assays were used (life technologies, USA). PCR reactions were performed in triplicate in a volume of 10 µL consisting of 2 µL of MilliQ water, 5 µL PCR master mix (Eurogentec, Seraing, Belgium), 0.5 µL assay mix (life technologies, USA), and 2.5 µL cDNA. Runs were performed by a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Data were analyzed with LightCycler® 480 SW 1.5 software (Roche, Basel, Switzerland) and the Fitpoints method. RNA data were normalized to Gapdh mRNA expression using $2^{-\Delta Cp}$ (Cp means crossing points). Undetectable Cp values of the genes of interest (>40) were interpreted as the maximum Cp value (40).

Immunohistochemistry

Sections (3 µm) of formalin-fixed and paraffin-embedded lung tissue were stained for ciliated- or club (previously Clara) secretory cells using standard immunohistochemical procedures. Briefly, slides were deparafinized and put in citrate buffer in a microwave oven for 15min. After cooling, slides were incubated in 0.3% H_2O_2 in PBS for 30 min. To visualize ciliated cells, slides were incubated with mouse-anti-acetylated α-tubulin at 1:10000 for 1h

(Sigma-Aldrich, Zwijndrecht, The Netherlands), which was detected by HRP-conjugated rabbit-anti-mouse antibody (1:200, Dako, Glostrup, Denmark) for 0.5h. A 0.05% diaminobenzidine (DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands) solution was used for color reaction. To visualize club secretory cells, a rabbit-anti-club cell 10 kD (CC10) antibody (1:6000, 1h, Millipore, Billerica, USA) was used as the first antibody and a horseradish peroxidase (HRP)-conjugated goat-anti-rabbit antibody (1:200, 0.5h) was used as the second antibody. A 0.05% DAB solution was used as chromogen. Numbers of α-tubulin-positive cells and CC10-positive cells were counted manually in all airways. The length of all airways was measured at the basal end of the airway epithelium using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, USA) and the total numbers of α-tubulin- and CC10-positive cells were expressed per mm airway.

Double staining of α-tubulin and CC10 was performed. Briefly, the slides were incubated with a rabbit-anti-CC10 antibody (1:6000, 1h), then a goat-anti-rabbit biotin-conjugated antibody (1:200, 30 min) and streptavidin-Alkaline Phosphatase (1:200, 30 min). After wash steps, slides were incubated in fast blue solution for 30 min to visualize color. The slides were subsequently incubated with a mouse-anti-acetylated α-tubulin antibody (1:10000, 1h) and a HRP-conjugated rabbit-anti-mouse secondary antibody (1:200, 0.5h) after wash steps, and then incubated in a 0.05% DAB staining solution for 10 min.

Statistical methods

Results obtained from qRT-PCR and IHC were expressed as median and range respectively. The effect of maternal smoking during pregnancy was investigated with a multiple linear regression using SPSS Statistics 22 (IBM, Amsterdam, The Netherlands). When residuals were not normally distributed, appropriate log10 or 1/x transformation of the data was performed. The interaction of the effect of smoking during pregnancy and the effect of sex was tested and not present, implying that the effect of prenatal smoking was similar in females and males. A value of $p<0.05$ was considered significant.

Results

Maternal smoking during pregnancy inhibited ciliated cell differentiation in offspring.

In order to investigate whether the previously observed HDM-induced goblet cell susceptibility in offspring from smoke-exposed mothers was due to the presence of more club cells in prenatally exposed neonates, we first investigated the mRNA expression of Scgb1a1, a marker for club cells. Figure 1A shows no difference in gene expression of Scgb1a1, which was supported by no difference in club cell numbers in both offspring groups, as analyzed by immunohistochemistry (Figure 2A-C). We then moved on to investigate gene expression of the ciliated cells (Foxj1) and neuroendocrine cells (Calca) as well. As shown in figure 1B, offspring from smoke-exposed mothers had lower expression of Foxj1 ($p<0.05$) than offspring from air-exposed mothers. Quantification of ciliated cells confirmed that numbers of ciliated cells were indeed lower in offspring from smoke-exposed mothers (Figure 2D-F, $p<0.001$). Gene expression of Calca (Figure 1C) was not different in both groups of offspring. As in the mouse, the club cell is thought to be the progenitor of the goblet cell, and as the ciliated cells and club cells are the two major cell types in the proximal airways of the mouse, increased numbers of club cells in offspring from smoke-exposed mothers were expected. Therefore, in a next experiment, the presence of club cells and ciliated cells were visualized in a double staining for CC10 (club cells) and α-tubulin (ciliated cells) and confirmed that both staining did not overlap, as shown in Figure 3. To investigate whether basal cells were affected by prenatal smoke exposure, expression of gene markers Trp-63 and Krt5, selectively expressed in basal cells were investigated. However, gene expression of both basal markers was not different in both groups (data not shown).

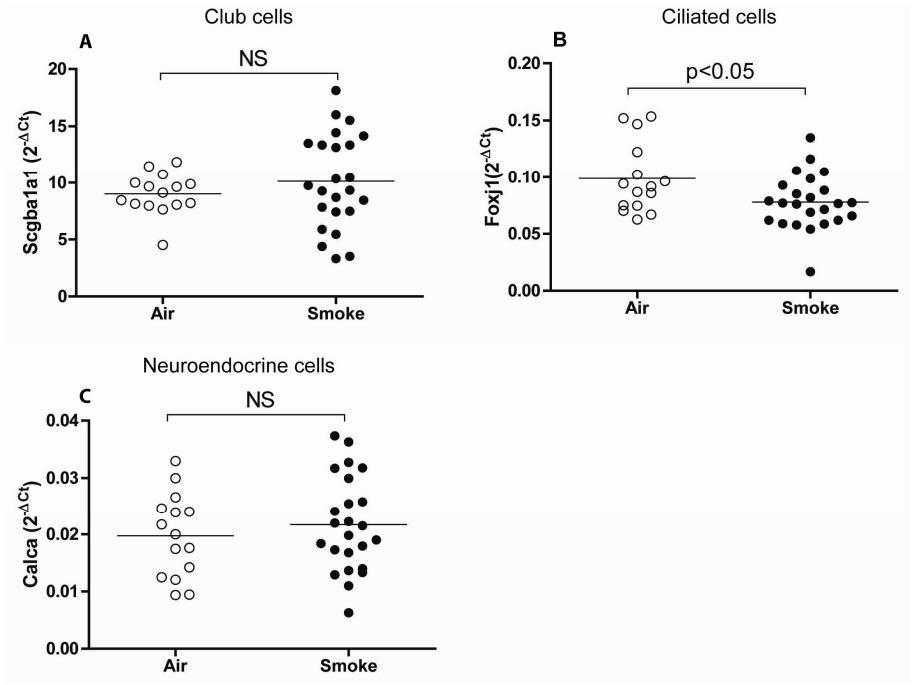

Figure 1. Expression of the differentiation markers Scgb1a1, a marker for club cells (A); Foxj1, a marker for ciliated cells (B) and Calca, a marker for neuroendocrine cells (C) was analyzed by real-time qPCR in RNA isolated from lung tissue. Data represent medians of expression in neonatal pups prenatally exposed to cigarette smoke or not.

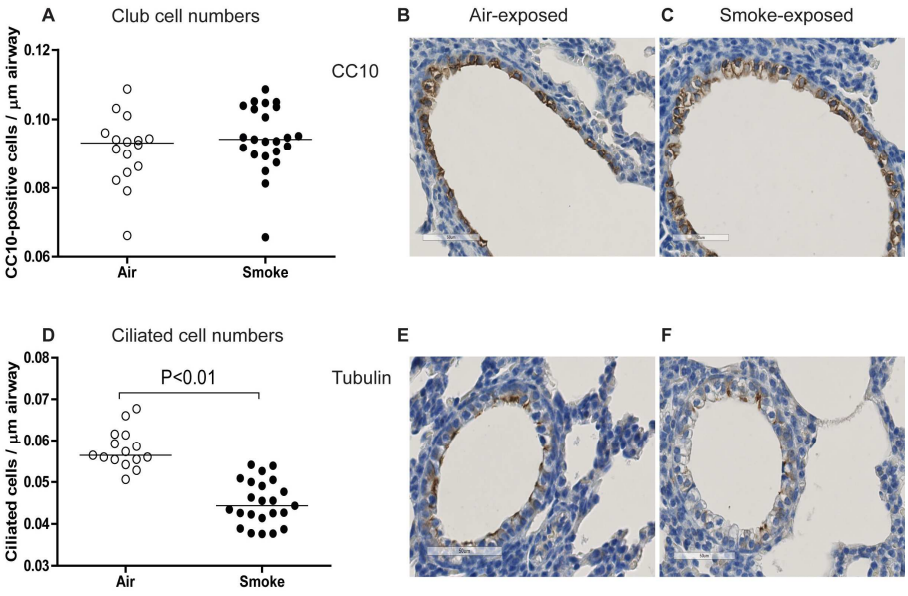

Figure 2. Club cell numbers (A) and immunohistochemical staining of CC10 (brown, B and C) or ciliated cell numbers (D) and immunohistochemical staining of α-tubulin (brown, E and F) in airways in lung tissue from offspring of air-exposed mothers (B and E) or smoke-exposed mothers (C, F). Original magnification 40×.

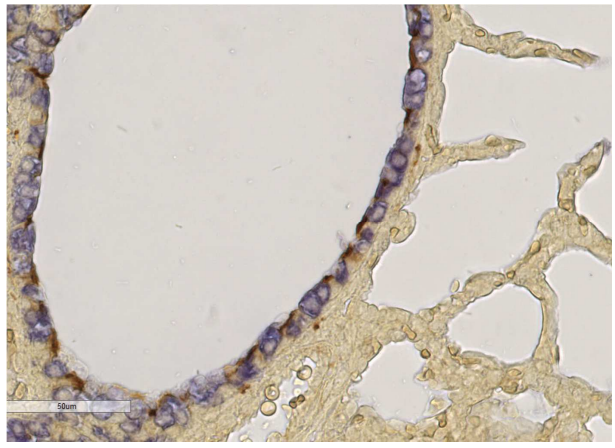

Figure 3. Representative example of a double staining for CC10 (blue) and α-tubulin (brown). Original magnification 40×.

Maternal smoking during pregnancy induced goblet cell-related gene expression in offspring.

In order to further explain the previously observed HDM-induced goblet cell susceptibility in offspring from smoke-exposed mothers, genes important in goblet cell transformation were investigated. As shown in Figures 4A and B, offspring from smoke-exposed mothers had higher expressions of Foxm1 ($p<0.05$) and Spdef ($p<0.001$). Expression of the genes Nkx2.1, Foxa2, Foxa3, Hoxa5 and Hoxb5, Muc5ac, that are essential to maintain club cell identity and/or inhibit Spdef function were either slightly decreased ($p=0.065$, Nkx2.1) or not affected (data not shown).

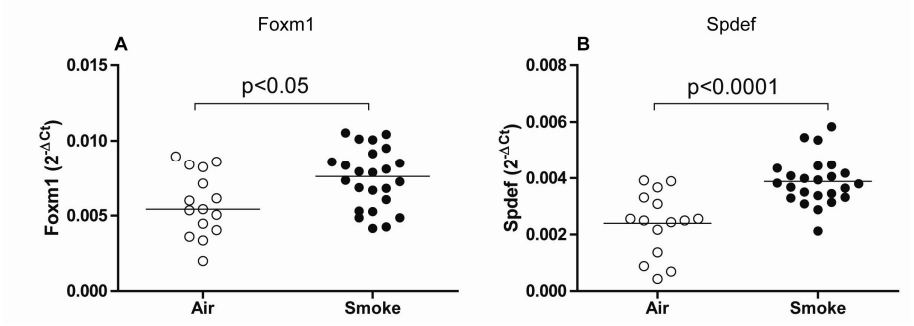

Figure 4. Expression of genes that drive secretory cell development. Expression of the transcription factors Foxm1 (A) and Spdef (B) was analyzed by real-time qPCR in RNA, isolated from lung tissue. Data represent medians of expression in neonatal pups prenatally exposed to cigarette smoke or not.

Maternal smoking during pregnancy affected Notch signaling pathway gene expression in offspring.

The Notch signaling pathway has been identified as a major regulator of specific cell fate in the developing and postnatal lung (Rock, et al. 2011; Tsao, et al. 2011). To further investigate whether Notch signaling was affected in prenatally smoke-exposed mice, gene expressions of Notch receptors 1, 2 and 3, and gene expression of Notch target genes Hey1, Hey2 and Hes1, which are important in airway epithelial cell differentiation, were investigated. As shown in Figure 5, offspring from smoke-exposed mothers had lower expression of Notch1 (Figure

5A) and a higher expression of the Notch target gene Hey1 (Figure 5B). The other members of this pathway that were investigated were not different (Notch2, Hey2 and Hes1, Figure 5C-E) between both groups, or were too minimally expressed (Notch3, data not shown).

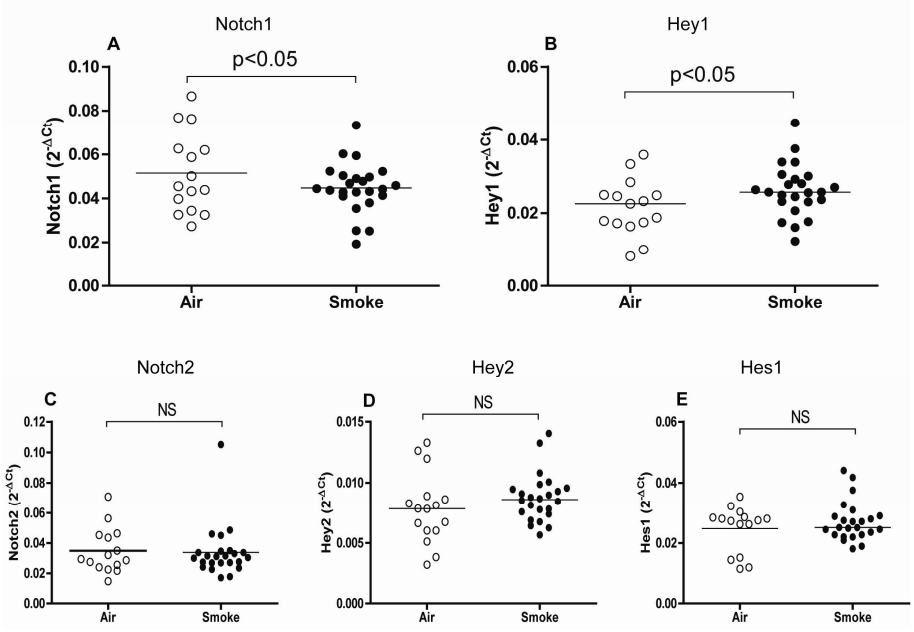

Figure 5. Expression of genes of the Notch signaling pathway. Expression of Notch1 (A), Hey1 (B), Notch2 (C), Hey2 (D) and Hes1 (E) was analyzed by real-time qPCR in RNA, isolated from lung tissue. Data represent medians of expression in neonatal pups, prenatally exposed to cigarette smoke or not.

Discussion

A number of studies by us and others have shown that smoking during pregnancy affects lung development and function in the offspring (Singh, et al. 2003; Gaworski, et al. 2004; Gaworski, et al. 2004; Blacquiere, et al. 2009; Blacquiere, et al. 2010; Haley, et al. 2011; Manoli, et al. 2012). In the present

study, the effect of maternal smoking during pregnancy on epithelial cell differentiation was investigated in neonatal offspring. Our three main observations in prenatally smoke-exposed offspring included: *lower* numbers of ciliated cells, *higher* expression of genes that are important in goblet cell differentiation, and lastly, altered expression of genes in the Notch signaling pathway.

This is the first study to show that prenatal smoke exposure is associated with downregulation of ciliated cells in the lung after birth. Ciliated cells are necessary for proper mucociliary clearance of particles and pathogens (Wanner, et al. 1996), and a decrease of ciliated cell numbers may account for a less effective clearance of pathogens from the lung. Indeed, epidemiological studies have shown that children born from a mother that smoked during pregnancy have a higher risk to suffer from airway infections (Broughton, et al. 2005). Our data is further supported by a recent *in vitro* study in which primary human bronchial epithelial cells were exposed to cigarette smoke extract (CSE) during differentiation at the air-liquid interface (Schamberger, et al. 2015). This CSE exposure was shown to reduce the number of ciliated cells, while it increased the number of club cells and goblet cells. In addition, Foxj1, a master regulator in ciliogenesis and responsible for cilia length (You, et al. 2004; Brekman, et al. 2014) was studied. However, no changes in Foxj1 or Foxj1 target gene transcription upon CSE exposure was found, indicating that CSE influenced Foxj1-independent processes crucial for ciliated cell fate, or affected ciliogenesis further downstream of Foxj1. In our study, lower numbers of ciliated cells were accompanied by lower expression of Foxj1. Foxj1 expression was recently shown to be directly regulated by the Wnt/β-catenin signaling pathway (Caron, et al. 2012). This is consistent with our earlier findings where we demonstrated less Wnt/β-catenin signaling in the lungs of neonatal offspring from smoke-exposed mothers (Blacquiere, et al. 2010).

Our finding that reduced numbers of ciliated cells were not accompanied by the presence of more CC10 positive club cells came somewhat as a surprise. As in the mouse, goblet cells have been shown to arise from club cells (Hayashi, et al. 2004; Kouznetsova, et al. 2007; Chen, et al. 2009), more club cells in offspring

from smoking mothers could have been one explanation for our previous observation regarding increased HDM-induced goblet cell susceptibility in this group. Interestingly, however, several studies in a naphthalene toxicity model have reported the presence of a new subset of CC10 positive secretory cells. These so-called variant club cells do not express cytochrome p450 (Cyp2f2), are naphthalene-resistant, and can self-renew and differentiate. They are located adjacent to neuroendocrine bodies of the airway (Hong, et al. 2001) or at the bronchoalveolar duct junctions (Giangreco, et al. 2002) to regenerate the damaged conducting airways (Reynolds, et al. 2000a; Reynolds, et al. 2000b). Whether prenatal smoke-exposure affects this cell population will be subject of further studies.

Another source for goblet cells could be the basal cell which in the mouse is mostly present in the trachea and the largest bronchi. Although expansion of the basal cell compartment is among the hallmark airway abnormalities of smokers and individuals with COPD (Demoly, et al. 1994; Khuri, et al. 2001), gene expression of two basal cell markers was not changed in offspring from smoking mothers.

Our second important observation was that prenatal smoke exposure increased expression of Foxm1, Spdef and decreased expression of Nkx2.1 (trend). Foxm1, a transcription factor of the Forkhead box family, plays important roles during embryonic development, monocyte/macrophage recruitment, DNA repair, surfactant production and angiogenesis (Kalinichenko, et al. 2001; Kalin, et al. 2008; Balli, et al. 2013). Moreover, Foxm1 is known to promote Spdef activity, a master transcription factor that regulates goblet cell differentiation in the airway epithelium (Park, et al. 2007). Therefore, higher expression of Foxm1 could have contributed to the higher expression of Spdef in offspring from smoke-exposed mothers. Interestingly, a trend for lower expression of Nkx2.1 was observed in prenatally exposed offspring. Nkx2.1 is a transcription factor that has been described to inhibit Spdef expression (Maeda, et al. 2011a), and therefore, a lower expression of Nkx2.1 could have facilitated the higher expression of Spdef in prenatally smoke-exposed offspring. We found no differences in Hoxa5 and Hoxb5 expression. In Hoxa5$^{-/-}$ mice, the loss of Hoxa5

function was shown to induce club to goblet cell transdifferentiation. This was a Foxa2-independent process, accompanied by increased activity of Notch signaling (Boucherat, et al. 2012).

Our finding regarding altered epithelial cell differentiation in prenatally smoke-exposed offspring does not support findings from a recent study in second hand smoke-exposed offspring (Singh, et al. 2013). In that study, no change in ciliated cells was found, whereas the numbers of club cells and CC10 gene expression were decreased. An explanation for these conflicting results could be that in the study from Singh *et al.* a different mouse strain was used (BALB/c), a different batch of cigarettes (2R1 research cigarettes), a different type of exposure (side stream instead of mainstream) and a different smoking protocol (6 h exposure versus 2 times 50 minutes). Side stream smoke (SS) contains different concentrations of toxic components than mainstream smoke. For instance, SS contains ten times greater levels of polycyclic aromatic hydrocarbons than mainstream smoke (Weinberg, et al. 1989).

Notch signaling has been reported to promote secretory cell development over ciliary cell fate (Rock, et al. 2009; Rock, et al. 2011). Here we show that prenatal exposure to cigarette smoke upregulates Hey1 expression. Hey1 is a Notch target gene which implies active Notch signaling in offspring from smoke-exposed mothers. Therefore, active Notch signaling could have contributed to lower ciliated cell development and increased expression of goblet cell gene markers.

In conclusion, our studies indicate that smoking during pregnancy in mice changes epithelial cell differentiation and therefore increases risk for asthma in the offspring at two levels: (*i*) by inhibiting ciliated cell differentiation, thereby increasing the risk to develop respiratory infections, which itself is a risk factor for asthma and (*ii*) by promoting secretory cell metaplasia through regulation of Foxm1, Spdef and Nkx2.1. These actions may be Notch signaling-related and provide insight into potential mechanisms underlying epidemiological observations on the association between maternal smoking and childhood or adolescent asthma.

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Conflict of interest

No competing interests declared.

Author contributions

JC performed the animal and lab experiments, collected data and conducted statistic analyses. He was also involved in the writing and review of the manuscript. JS performed experiments in the lab and reviewed and edited the manuscript. MRL and WK supervised the experimental work and reviewed the manuscript. KvS performed experiments and reviewed the manuscript. XH and WT reviewed and edited the manuscript. SKE was involved in the methodology development of the smoke experiment and reviewed and edited the manuscript, MH acquired funding, designed and supervised the experiment, was involved in the writing and editing of the manuscript.

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